

Metastasis inhibition after proton beam, β - and γ -irradiation of melanoma growing in the hamster eye*

Bożena Romanowska-Dixon¹, Martyna Elas^{2✉}, Jan Swakoń³, Urszula Sowa³,
Marta Ptaszek³, Małgorzata Szczygieł², Martyna Krzykawska², Paweł Olko³
and Krystyna Urbańska²

¹Department of Ophthalmology and Ocular Oncology, Jagiellonian University Medical College, Kraków, Poland; ²Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland; ³The H. Niewodniczanski Institute of Nuclear Physics, Polish Academy of Sciences, Kraków, Poland

Standard ocular tumor treatment includes brachytherapy, as well as proton therapy, particularly for large melanoma tumors. However, the effects of different radiation types on the metastatic spread is not clear. We aimed at comparing ruthenium (¹⁰⁶Ru, emitting β electrons) and iodine (¹²⁵I, γ -radiation) brachytherapy and proton beam therapy of melanoma implanted into the hamster eye on development of spontaneous lung metastases. Tumors of Bomirski Hamster Melanoma (BHM) implanted into the anterior chamber of the hamster eye grew aggressively and completely filled the anterior chamber within 8–10 days. Metastases, mainly in the lung, were found in 100% of untreated animals 30 days after enucleation. Tumors were irradiated at a dose of 3–10 Gy with a ¹⁰⁶Ru plaque and at a dose of 6–14 Gy using a ¹²⁵I plaque. The protons were accelerated using the AIC-144 isochronous cyclotron operating at 60 MeV. BHM tumors located in the anterior chamber of the eye were irradiated with 10 Gy, for the depth of 3.88 mm. All radiation types caused inhibition of tumor growth by about 10 days. An increase in the number of metastases was observed for 3 Gy of β -irradiation, whereas at 10 Gy an inhibition of metastasis was found. γ -radiation reduced the metastatic mass at all applied doses, and proton beam therapy at 10 Gy also inhibited the metastatic spread. These results are discussed in the context of recent clinical and molecular data on radiation effects on metastasis.

Key words: metastases, melanoma, ocular, proton beam, iodine-125, ruthenium-106

Received: 23 April, 2013; **accepted:** 05 June, 2013; **available on-line:** 01 July, 2013

INTRODUCTION

Particle radiotherapy, such as proton beam, yields promising clinical results worldwide, but the mechanisms of its action are still unclear. Ocular melanoma is one of the tumors treated by protons with excellent clinical results (Fuss *et al.*, 2001; Höcht *et al.*, 2004; Gragoudas, 2006). The advantage of particle beams over photons is due to the physical characteristics and results in superior distribution of the radiation dose, which allows to spare normal tissue close to the target (Levin *et al.*, 2005; Fokas *et al.*, 2009). Proton beam therapy is also applied in other cancer treatments (Mizumoto *et al.*, 2010; Mizumoto *et al.*, 2011; Lorentini *et al.*, 2012). However, the effects of particle beams on metastatic spread of cancer are not yet

well understood. There are only a few publications on this subject. It was shown that particle beams might inhibit cellular metastatic potential (Takahashi *et al.*, 2003). Proton ion irradiation was also demonstrated to decrease cell migration and invasion in a dose-dependent manner and strongly inhibit matrix metalloproteinase-2 activity in two preclinical models (Ogata *et al.*, 2005). These results suggest that particle irradiation suppresses metastatic potential at a low dose, whereas another report shows that photon irradiation promotes cell migration and invasive capabilities at a low dose level.

Enhancement of migratory properties of different tumor cell lines were found after 1–3 Gy of gamma irradiation (Wild-bode *et al.*, 2001; Goetze *et al.*, 2010). Several factors associated with increased migratory properties have been found to be induced by gamma irradiation, such as HGF receptor/c-Met expression (Qian *et al.*, 2003), integrin expression (Onoda *et al.*, 1992; Goetze *et al.*, 2010) or MMP-2 activity (Wild-bode *et al.*, 2001; Park *et al.*, 2006; Martinou *et al.*, 2011). In particular there are suggestions that it is the interaction with tumor microenvironment that influences cell migration and invasiveness (Ohuchida 2004; Madani *et al.*, 2008).

These findings taken together indicate that ion beam radiotherapy may be superior to conventional photon beam therapy in possible preventive effects on tumor cell metastasis (Girdhani *et al.*, 2013). In our animal model we can observe both immediate effects, such as inhibition of the growth of the radiation treated tumor, and long-term effects of the therapy, such as reduction in the number of metastases (Urbańska *et al.*, 2000; Matuszak *et al.*, 2000).

The goal of this study was to compare the effects of β - and γ -radiation using brachytherapy and proton beam radiation of melanoma tumor growing in the eye on the development of metastases in the lung. In this study we show, for the first time, a substantial inhibition of lung metastasis after a single dose of 10 Gy (ie. 11.1 CGE Cobalt Grey Equivalent) of proton beam irradiation of the melanoma tumor growing in the hamster eye.

✉ e-mail: martyna.elas@uj.edu.pl

*Presented at 40th Jubilee Winter School of the Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University "Contemporary insights into cancer. Risk, perspectives, expectations", February 16–21, 2013, Zakopane, Poland.

Abbreviations: BHM, bomirski hamster melanoma; CGE, cobalt grey equivalent; HGF, hepatocyte growth factor; MMP2, matrix metalloproteinase 2; PMMA, poly(methyl methacrylate); SOB, spread out bragg peak.

MATERIALS AND METHODS

Animals and tumors. The total of 60 female hamsters, weighting 80–120g, derived from the breeding facility at the University of Silesia, Katowice, Poland, were maintained on the standard laboratory diet with free access to drinking water. The study was performed in compliance with European policies and was approved by the Bioethics Committee for Animal Experiments (no 280/96, 384/99 and 35/2011 from April 20th 2011). A spontaneous skin melanoma, Bomirski Hamster Melanoma (Bomirski *et al.*, 1988), was used as a tumor model. Tumor implantation was performed as described earlier (Kukielczak *et al.*, 1999; Urbanska *et al.*, 2000; Romanowska-Dixon *et al.*, 2001; Romanowska-Dixon *et al.*, 2003). Briefly, tumor fragments were implanted microsurgically into the anterior chamber of the hamster eye. Six to nine days later tumors were visible, growing in the anterior chamber on the surface of the iris as well demarcated, dome-shaped single nodules. To non-invasively monitor the tumor growth in the hamster eye *in vivo* we estimated two dimensions of the tumor on the surface of the iris, applying a microscopic ruler. This approach assumes that assessment of only two dimensions in the plane of the iris reflects tumor growth adequately, as the growth in the depth dimension is limited by the anterior chamber volume. Two perpendicular diameters “a” and “b” of the tumor were measured each day and the mean tumor dimension was calculated ($\sqrt{a \times b}$) in mm. Eyeballs were enucleated when the anterior chamber was completely filled with the tumor. Thirty days after the enucleation animals were sacrificed, and internal organs were examined for metastases, clearly visible as black dots. Lungs were isolated and weighted.

Brachytherapy. Tumors were irradiated at a dose of 3, 6 and 10 Gy with a ^{106}Ru plaque (model Rue. EBB, 11.8 Mbq, BEBIG GmbH, Germany) at 0.017

Gy/min and with a ^{125}I plaque (BEBIG GmbH, Germany) at 0.012 Gy/min. All radiation exposures were preceded by injections of Vetbutal (36 mg/kg body weight). Doses were fractionated into 4 equal fractions administered every 24 hrs. The time of irradiation for a single fraction was from 62 to 294 min. ^{106}Ru emitted electrons have the energy of 3.54 MeV and ^{125}I is a source of γ -rays with the energy of 35.4 keV.

Proton beam. Protons for the irradiation experiments were accelerated using the AIC-144 isochronous cyclotron, operating at 60 MeV, which was designed and constructed at IPJ PAN (Kraków, Poland). The beam delivery system, the formation of a primary proton beam, was described by Bakewicz and Swakon (Bakewicz *et al.*, 2003; Swakon *et al.*, 2010).

The proton beam had to be specially formed for the irradiation purposes. The uniform lateral dose distribution was realized by a passive scattering with single tantalum foil (Swakon *et al.*, 2010, Michalec *et al.*, 2010). The Spread Out Bragg Peak (SOBP) was formed by a dedicated rotating modulator wheel, made of PMMA. The proton beam range was controlled by a PMMA range shifter or a set of PMMA plates with different thickness.

For the irradiation of hamster eyeballs the proton beam was configured to irradiate only the anterior chamber of the hamster eye. The proton beam has been prepared to penetrate the tissue to a depth of 3.88 mm (4.07 mm in water). The diameter of the final collimator was equal to 7 mm in order to irradiate only the eyeball area with a small margin. The transversal profiles were measured in air using a one dimensional scanner with diode and presented in the inset of Fig. 1C.

The proton beam monitoring system and the beam dosimetry. The set of ion chambers with dedicated Ergen electrometers was used for monitoring the proton beam parameters. The proton beam intensity has been measured by two PTW transmission ionization chambers type 7862. During irradiation the type 7862 ionization chambers carried out the function of a dose monitor. The time and spatial stability of the beam was controlled on-line by a six sector dedicated ion chamber, which consisted of four ion chambers in the form of quarters, a circle ion chamber and a ring one.

The PTW UNIDOS electrometer and the Markus chamber PTW type 23343 have been used to calibrate the dose. Dose measurements were performed in the middle of SOBP using the solid phantom (PMMA). Overall uncertainty of dosimetry was about 3%, the precision of dose delivery was better than 0.5%. The average dose rate of the proton beam during irradiation was between 0.07 and 0.08 Gy/s.

Hamster eye positioning and proton beam irradiation. Prior to the animal experiments, a phantom of the hamster head was used to develop the animal positioning system and hamster eye positioning. The analysis took into

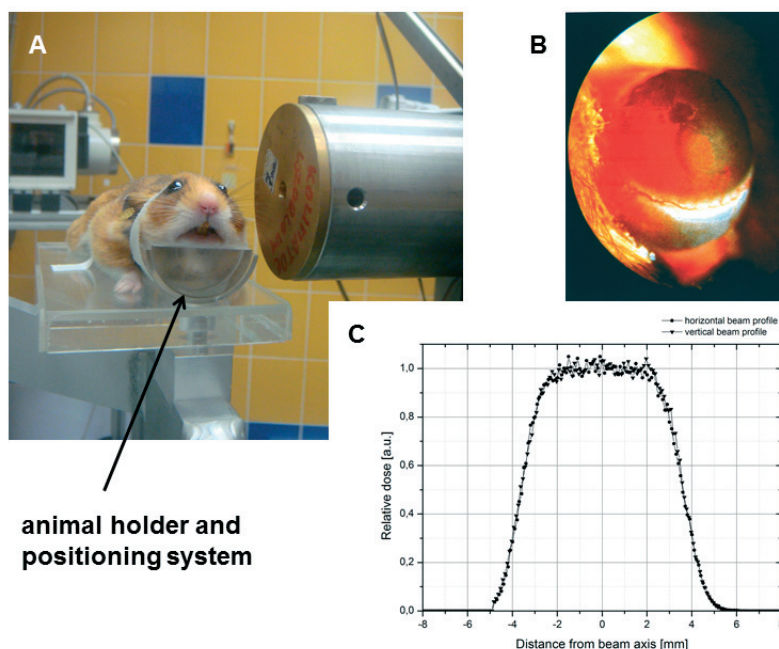


Figure 1. Hamster eye irradiation with proton beam.

(A) Hamster in the animal positioning system, (B) hamster eye with the tumor, magnification 10x and (C) proton beam dose depth distribution and lateral profiles of the beam used for hamster eye irradiation.

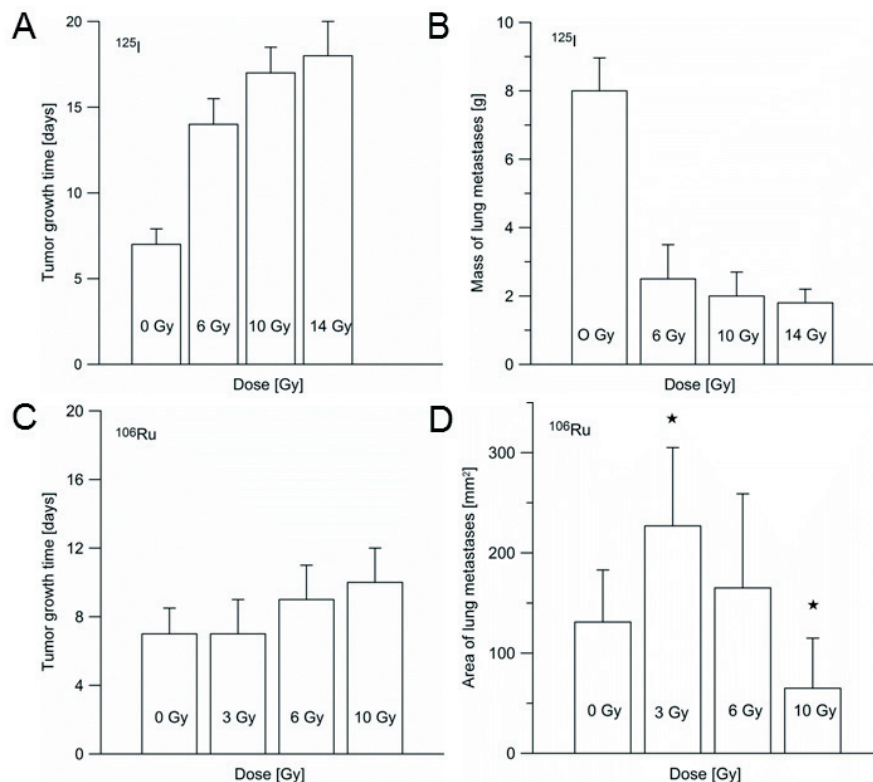


Figure 2. The effects of γ - and β -irradiation of the BHM melanoma tumor growing in the hamster eye.

(A) Growth inhibition of primary tumor implanted in the eye by ^{125}I irradiation. (B) Inhibition of lung metastasis after a fractionated dose of 6–14 Gy of ^{125}I irradiation of BHM melanoma tumors growing in the hamster eye. (C) ^{106}Ru slightly affected tumor growth in the eye. (D) The effect of fractionated doses of ^{106}Ru irradiation on lung metastasis. The experimental group consisted of 5–8 of animals. Asterisks indicate statistical significance of $p < 0.05$ in comparison with control.

account the protruding shape of the hamster eye and the size of the anterior chamber. The animal positioning system consisted of an animal holder, attached to the frame of the 3D positioning system, movable at 0.01 mm steps. A laser positioning subsystem and entrance field simulation light have been used for an accurate hamster eye positioning in the proton beam.

A single dose of 10 Gy (11 CGE) has been delivered within a maximum irradiation time of 150 s. Animals were anesthetized for about 30 min, for both the positioning and irradiation, using 100 mg/kg ketamine (Bioketan, Vetoquinol, Biowet, Poland) and 10 mg/kg xylazine (Sedazin, Biowet, Poland).

Statistical analysis. The significance of the difference between the experimental groups was determined using Student *t*-test, with $p < 0.05$.

RESULTS AND DISCUSSION

Growth inhibition of the primary tumor

^{125}I irradiation led to inhibition of the tumor growth in the eye from 7 days in untreated animals to 14–17 days in animals irradiated with fractionated doses between 6–14 Gy (Fig. 2A). ^{106}Ru brachytherapy did not affect the primary tumor growth (data not shown). After several days, some regrowth of the primary tumor was observed. The inhibition after γ -radiation was similar to that achieved after 10 Gy of proton beam irradiation (Fig. 3B). It has to be pointed out that different radiation dose rates have been used: 0.017 Gy/min for ^{106}Ru , 0.012 Gy/min for ^{125}I and 4.2 Gy/min for proton beam irradiation. Therefore a direct comparison of the effects is not possible.

Enhancement of metastasis by very low doses of beta-radiation

Treatment with γ -irradiation strongly inhibited metastatic spread, as seen in Fig. 2B, however, it seems that the lowest dose of 3 Gy of β -radiation caused an increase in lung metastasis. The largest applied dose of 10 Gy of β -radiation led to inhibition of metastasis (Fig. 2C).

Our previous studies have shown that BHM melanoma growing in the hamster eye is heavily vascularized, and that this vascular network is highly pathological, with densely distributed, tortuous, and irregular vessels, with many sprouts and embolisms (Romanowska-Dixon *et al.*, 2001). One may speculate

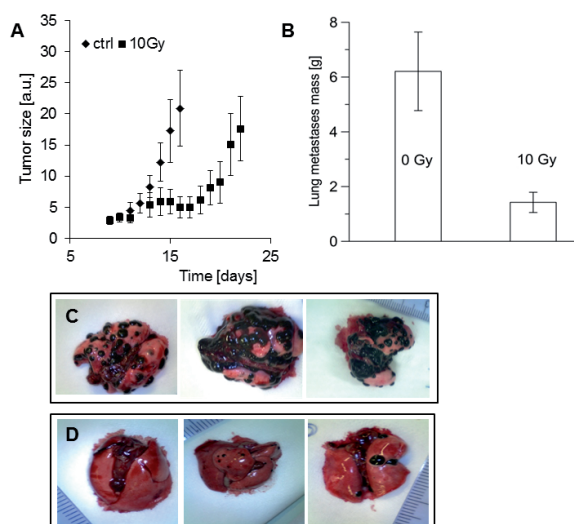


Figure 3. (A) Inhibition of BHM melanoma tumor growing in the hamster eye; irradiated with a proton beam at a single dose of 10 Gy ($n=7$, black square), in comparison with untreated control ($n=6$, black diamond). (B) The mass of metastases to the lung decreased 4.35 times after proton beam irradiation (10 Gy) of BHM melanoma tumor growing in the hamster eye ($p=0.0052$). Average mass with SEM is shown. The number of control animals was 6, and the number of irradiated animals was 7. Representative isolated lungs with metastases from untreated (C) and irradiated (D) animals.

that irradiation of such tissue will lead to destruction of endothelial cells, and subsequently to leakage of vasculature, thus promoting metastatic spread. One of the mechanisms involved in the endothelial cell death after radiation is activation of the ceramide-sphingomyelin pathway, although usually induced at the level of 10 Gy dose (Kolesnick & Fuks, 2003; Fuks & Kolesnick, 2005).

The clinical reports justifying the use of short-range electron emitting brachytherapy did not demonstrate any enhancement of metastasis (Lommatzsch *et al.*, 2000). There was a suggestion, however, to treat patients with possibly low dose-rate plaques (Kleineidam & Guthoff 1994). The randomized trial of 125I brachytherapy did not find any differences in the metastasis rates (COMS 2006).

Inhibition of metastasis by proton beam irradiation of the primary tumor

A single dose of 10 Gy of proton beam irradiation delayed the growth of the BHM melanoma in the hamster eye by 10 days (Fig. 3B). Albeit the inhibition of the primary tumor growth was moderate, proton therapy strikingly reduced the mass of the metastases in the lungs in comparison with untreated animals. On average, 10 Gy of proton irradiation diminished the mass of metastases 4.35 times, even though there was a significant spread between individual animals (Fig. 3A, C, D). These results are in agreement with data for osteosarcoma published by Ogata *et al.* (2005). Proton ion irradiation decreased cell migration and invasion in a dose-dependent manner and strongly inhibited matrix metalloproteinase-2 activity in highly aggressive HT1080 human *fibrosarcoma* cells *in vitro* and significantly decreased the number of pulmonary metastases in mouse *osteosarcoma in vivo* (Ogata *et al.*, 2005). Similarly, it was shown that in *in vitro* models cell properties such as adhesion, migration, invasion, and the expression level or activity of molecules related to metastasis such as $\alpha V\beta 3$, $\beta 1$ integrin, and MMP-2 were decreased after small doses of proton beam treatment (Takahashi *et al.*, 2003).

Suppression of the growth of spontaneous metastases observed after proton beam therapy in hamsters may stem from numerous mechanisms, such as inhibition of cell motility, adhesion, and invasion, or perturbation of cell death pathways. To fully address these issues, we will continue to study these mechanisms on the cellular, molecular and submolecular level.

CONCLUSIONS

1. Small, sublethal doses of β -irradiation might increase the metastatic spread.
2. Proton beam irradiation at 10 Gy inhibited the growth of melanoma tumor in the hamster eye and diminished number of metastases in the lung more than four times.
3. The already established infrastructure (modernized AIC-144 cyclotron, the beam delivery system, the therapy room) as well as the equipment for the beam control and monitoring and the human eye positioning systems, prepared for treatment of human patients, were adapted and developed for irradiation of a very small, 4–6 mm in diameter, hamster eye.
4. The mechanisms of tumor growth inhibition after proton beam irradiation need further explanation.

Acknowledgments

This work was partly supported by the Ministry of Science and Higher Education grant IAEA/PM5/2007, and National Science Center, Poland grant DEC-2012/07/B/NZ4/01657. The IFJ PAN facility benefited from the IAEA Technical Cooperation Program (POL/6/009).

REFERENCES

- Bakewicz E, Budzanowski A, Taraszkiewicz R (2003) AIC-144 cyclotron: present status. *Nukleonika* **48**: 117–121.
- Bomirski A, Slominski A & Bigda J (1988) The natural history of a family of transplantable melanomas in hamsters. *Cancer Metastasis Rev* **7**: 95–118.
- COMS (2006) The COMS randomized trial of iodine 125 brachytherapy for choroidal melanoma: V. Twelve-year mortality rates and prognostic factors: COMS report No. 28. *Archives Ophthalmol* **124**: 1684–1693.
- Fokas E, Kraft G, An H, Engenhardt-Cabillic R (2009) Ion beam radiobiology and cancer: time to update ourselves. *Biochim Biophys Acta* **1796**: 216–229.
- Fuks Z, Kolesnick R (2005) Engaging the vascular component of the tumor response. *Cancer Cell* **8**: 89–91.
- Fuss M, Loreda LN, Blacharski P, Grove RI, Slater JD (2001) Proton radiation therapy for medium and large choroidal melanoma: preservation of the eye and its functionality. *Int J Radiation Oncol, Biol, Physics* **49**: 1053–1059.
- Girdhani S, Sachs R, Hlatky L (2013) Biological effects of proton radiation: what we know and don't know. *Radiation Res* **179**: 1–16.
- Goetze K, Scholz M, Taucher-Scholz G, Mueller-Klieser W (2010) Tumor cell migration is not influenced by p21 in colon carcinoma cell lines after irradiation with X-ray or (^{12}C) heavy ions. *Radiation Environ Biophys* **49**: 427–435.
- Gragoudas ES (2006) Proton beam irradiation of uveal melanomas: the first 30 years. The Weisenfeld Lecture. *Investigative Ophthalmol Visual Sci* **47**: 4666–4673.
- Höcht S *et al.* (2004) Proton therapy of uveal melanomas in Berlin. 5 years of experience at the Hahn-Meitner Institute. *Strahlentherapie und Onkologie* **180**: 419–424.
- Kleineidam M, Guthoff R (1994) Possible effects of radiobiological parameters on metastatic spread of uveal melanomas treated with ^{106}Ru plaques. *Ger J Ophthalmol* **3**: 22–25.
- Kolesnick R, Fuks Z (2003) Radiation and ceramide-induced apoptosis. *Oncogene* **22**: 5897–5906.
- Kukielczak B, Romanowska B, Bryk J (1999) Gamma radiation and MC540 photosensitization of melanoma in the hamster's eye. *Melanoma Res* **9**: 115–124.
- Levin WP, Kooy H, Loeffler JS, DeLaney TF (2005) Proton beam therapy. *Brit J Cancer* **93**: 849–854.
- Lommatzsch PK, Werschnik C, Schuster E (2000) Long-term follow-up of Ru-106/Rh-106 brachytherapy for posterior uveal melanoma. *Graefes Archive Clin Exp Ophthalmol* **238**: 129–137.
- Lorentini S, Amichetti M, Spiazzi L, Tonoli S, Magrini SM, Fellin F, Schwarz M (2012) Adjuvant intensity-modulated proton therapy in malignant pleural mesothelioma. A comparison with intensity-modulated radiotherapy and a spot size variation assessment. *Strahlentherapie und Onkologie: Organ der Deutschen Röntgengesellschaft* **188**: 216–225.
- Madani I, Neve W De, Mareel M (2008) Does ionizing radiation stimulate cancer invasion and metastasis. *Bull Cancer* **95**: 292–300.
- Martinou M, Giannopoulou E, Malatara G, Argyriou A, Kalofonos HP, Kardamakis D (2011) Ionizing radiation affects epidermal growth factor receptor signalling and metalloproteinase secretion in glioma cells. *Cancer Genomics Proteomics* **8**: 33–38.
- Matuszak Z, Romanowska-Dixon B, Skwara A, Urbanska K, Starzycka M (2000) The effects of irradiation with ^{106}Ru and ^{125}I on the growth and metastases of experimental melanoma. *Curr Top Biophys* **24**: 79–82.
- Michalec B, Swakoń J, Sowa U, Ptaszekiewicz M, Cywicka-Jakiel T, Olko P (2010) Proton radiotherapy facility for ocular tumors at the IFJ PAN in Kraków Poland. *Applied radiation and isotopes: including data, instrumentation and methods for use in agriculture, industry and medicine*. **68**: 738–742.
- Mizumoto M *et al.* (2010) Clinical results of proton-beam therapy for locoregionally advanced esophageal cancer. *Strahlentherapie und Onkologie* **186**: 482–488.
- Mizumoto M *et al.* (2011) Proton beam therapy for hepatocellular carcinoma: a comparison of three treatment protocols. *Int J Radiation Oncol, Biol, Physics* **81**: 1039–1045.
- Ogata T *et al.* (2005) Particle irradiation suppresses metastatic potential of cancer cells. *Cancer Res* **65**: 113.
- Ohuchida K (2004) Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions. *Cancer Res* **64**: 3215–3222.

- Onoda J, Piechocki M, Honn K (1992) Radiation-induced increase in expression of the α 5 β 3 integrin in melanoma cells: effects on metastatic potential. *Radiation Res* **130**: 281–288.
- Park C-M, Park M-J, Kwak H-J, Lee H-C, Kim M-S, Lee S-H, Park I-C, Rhee CH, Hong S-I (2006) Ionizing radiation enhances matrix metalloproteinase-2 secretion and invasion of glioma cells through Src/epidermal growth factor receptor-mediated p38/Akt and phosphatidylinositol 3-kinase/Akt signaling pathways. *Cancer Res* **66**: 8511–8519.
- Qian L-W, Mizumoto K, Inadome N, Nagai E, Sato N, Matsumoto K, Nakamura T, Tanaka M (2003) Radiation stimulates HGF receptor/c-Met expression that leads to amplifying cellular response to HGF stimulation via upregulated receptor tyrosine phosphorylation and MAP kinase activity in pancreatic cancer cells. *Int J Cancer* **104**: 542–549.
- Romanowska-Dixon B, Urbanska K, Elas M, Cichocki T (2003) The histology of pigmented Bomirski melanoma growing in the hamster eye — preliminary results. *Cellular Mol Biol Lett* **5**: 849–856.
- Romanowska-Dixon B, Urbanska K, Elas Martyna, Pajak Stanislaw, Zygulska-Mach H, Miodonski A, Urbanska K (2001) Angiomorphology of the pigmented Bomirski melanoma growing in hamster eye. *Annals Anatomy* **183**: 559–565.
- Swakon J *et al.* (2010) Facility for proton radiotherapy of eye cancer at IFJ PAN in Krakow. *Radiation Measurements* **45**: 1469–1471.
- Takahashi Y *et al.* (2003) Heavy ion irradiation inhibits *in vitro* angiogenesis even at sublethal dose. *Cancer Res* **63**: 4253.
- Urbanska K *et al.* (2000) Experimental ruthenium plaque therapy of amelanotic and melanotic melanomas in the hamster eye. *Melanoma Res* **10**: 26–35.
- Wild-Bode C, Weller M, Rimner A, Dichgans J, Wick W (2001) Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. *Cancer Res* **61**: 2744–2750.